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RelA Is a Potent Transcriptional Activator of the CD28 Response Element within the Interleukin 2 Promoter

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T-cell activation requires two different signals. The T-cell receptor's recognition of a specific antigen on antigen-presenting cells provides one, and the second signal comes from costimulatory molecules such as CD28. In contrast, T cells that are stimulated with antigen in the absence of the CD28 costimulatory signal can become anergic (nonresponsive). The CD28 response element (CD28RE) has been identified as the DNA element mediating interleukin 2 (IL-2) gene activation by CD28 costimulation. Our previous work demonstrates that the Rel/NF-κB family proteins c-Rel, RelA (p65), and NFKB1 (p50) are involved in the complex that binds to the CD28RE. We also showed that c-Rel, but not NFKB1 (p50), can bind to the CD28RE and activate CD28RE-driven transcription in cotransfection assays. However, the role of RelA (p65) in CD28 signaling has not yet been addressed. We provide evidence that RelA (p65) itself bound directly to the CD28RE of the IL-2 promoter and other lymphokine promoters. In addition, RelA (p65) was a potent transcriptional activator of the CD28RE in vivo. We show that a RelA (p65)–c-Rel heterodimer bound to the CD28RE and synergistically activated the CD28RE enhancer activity. We also demonstrate that activated Raf-1 kinase synergized with RelA (p65) in activating the CD28RE enhancer activity. Interestingly, a soluble anti-CD28 monoclonal antibody alone, in the absence of other stimuli, also synergized with RelA (p65) in activating the CD28RE. Furthermore, we show that RelA (p65) activated expression of the wild-type IL-2 promoter but not the CD28RE-mutated IL-2 promoter. A combination of RelA (p65) and NFKB1 (p50) also activated the IL-2 promoter through the CD28RE site. These results demonstrate the functional regulation of the CD28RE, within the IL-2 promoter, by Rel/NF-κB transcription factors.

CD28 is a 44-kDa homodimeric receptor on the surface of 50 to 70% of $CD8^+$ and 95% of $CD4^+$ human T lymphocytes (3, 15) which interacts with the B7-1 and B7-2 ligands on antigen-presenting cells (for reviews, see references 31 and 47). Both the CD28 and B7 genes are members of the immunoglobulin superfamily. In the absence of CD28 costimulation, T cells activated by antigen alone may become anergic (nonresponsive) (24, 28, 72). When the soluble CTLA-4 (a CD28 homolog) fusion protein CTLA-4–immunoglobulin is used, it blocks the CD28 costimulatory signal and prolongs survival of transplanted organs in animal studies (41, 48).

CD28 stimulation displays two major effects. First, CD28 signaling enhances the stability of multiple lymphokine transcripts and increases their secretion (45, 46). Second, the combination of CD28, phorbol 12-myristate 13-acetate (PMA), and ionomycin (or anti-CD3) stimulation of T cells provides significant induction of interleukin 2 (IL-2) transcription via the CD28 response element (CD28RE) within the IL-2 promoter compared with that provided by PMA plus ionomycin (or anti-CD3) treatment without CD28 stimulation (20, 80). The CD28RE, containing a sequence similar but not identical to the κB site, is positioned between nucleotides −164 and −154 within the IL-2 promoter (20). This conserved CD28RE has been found in the promoters of many cytokine genes, including the granulocyte-macrophage colony-stimulating factor (GM-CSF), IL-3, gamma interferon (IFN-γ), and IL-2 genes (21, 65). Our previous results indicate that all three Rel family proteins (c-Rel, RelA [p65], and NFKB1 [p50]) are in the complex that bind to the CD28RE (10, 23).

Rel/NF-κB family transcription factors (reviewed in references 6, 66, and 74) include RelA (p65), RelB, c-Rel, NFKB1 (p105/p50), and NFKB2 (p100/p52) and are involved in immunological responses, cellular proliferation, and programmed cell death (1). These subunits share the Rel homology domain, a homologous 300-amino-acid stretch, in their amino-terminal regions. The Rel/NF-κB family of transcription factors activate gene expression by binding directly to the κB sites. The interplay of different combinations of these transcription factors determines the specific activation or suppression of target genes (32, 38, 53). These transcriptional factors can be constitutively expressed, induced, or both, depending on the tissue type (42). Although these family proteins may work together on a specific gene, their expression or activation may be regulated differently (16).

RelA (p65), a ubiquitously expressed protein, can bind to the κB site alone (61, 78). In addition, RelA (p65) is shown to be the subunit responsible for providing transcriptional activity of the NF-κB (RelA [p65]-NFKB1 [p50]) heterodimeric complex (62). The endogenous RelA (p65) homodimer can be induced by phorbol ester treatment in Jurkat T cells, in which the homodimeric RelA (p65) can be recognized and regulated by IκBα (22), an inhibitor of the Rel/NF-κB family proteins in the cytosol (5, 77). In addition to being regulated by IκBα, RelA (p65) is regulated by two other ankyrin repeat-containing proteins, NFKB1 (p105) and NFKB2 (p100), which interact with RelA (p65) to form an autoregulatory loop that tightly controls the activation and expression of the proteins in the

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NF-κB complex (7, 71). Furthermore, the demonstration of cross talk between RelA (p65) and basic leucine zipper region-containing proteins to activate their respective genes highlights the biological importance of RelA (p65) in regulating different genes (69).

We previously showed that CD28 signaling causes a sustained reduction of IκBα and leads to enhanced nuclear translocation of the Rel/NF-κB transcription factors (10, 37). Since RelA (p65) is contained in the CD28-responsive complex (CD28RC), we decided to study the role of RelA (p65) in CD28 signal transduction. We demonstrate that RelA (p65) can bind to the CD28RE in vivo and in vitro, with functional activation of the CD28RE in vivo. In addition, the RelA (p65)–c-Rel heterodimer can bind to the CD28RE and synergistically activate its enhancer activity. We show that activated Raf-1 kinase synergizes with RelA (p65) in activating the CD28RE enhancer activity. Interestingly, an anti-CD28 monoclonal antibody (MAb) alone, in the absence of other stimuli, also can synergize with RelA (p65) in activating the CD28RE. Moreover, activation of the CD28RE by RelA (p65) is also demonstrated in the wild-type IL-2 promoter but not in the CD28RE-mutated IL-2 promoter. A combination of RelA (p65) and NFKB1 (p50) is a strong transcriptional activator of the wild-type IL-2 promoter which works specifically through the CD28RE site. To our knowledge, this is the first evidence indicating that the CD28RE site is a functional Rel/NF-κB response element within the IL-2 promoter.

MATERIALS AND METHODS

Cells, reagents, and antibodies. Isolation of human peripheral blood T lymphocytes and preparation of nuclear extracts were performed as described previously (23). Human lymphoblastoid Jurkat T cells (clone J.LEI or E6-1) were grown in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (Gibco), 2 mM glutamine, and 1,000 U of penicillin-streptomycin (Sigma) per ml. One day before transfection, Jurkat T cells were split 1:2 by adding an equal volume of fresh medium. The purified anti-CD28 MAb (clone 9.3; used at 100 ng/ml) was kindly provided by Carl June (Naval Medical Research Institute, Bethesda, Md.). The anti-CD28 ascites (clone 9.3; used at a dilution of 1:2,000) was generously provided by Bristol-Myers Squibb Pharmaceutical Research Institute (Seattle, Wash.). PMA (Sigma) was used at a concentration of 10 ng/ml for stimulation. Rabbit antisera against RelA (p65) (antibody [Ab] 1226 or 1207), c-Rel (Ab 265) (60), and Bcl-3 (Ab 1348) and a nonspecific viral antigen (Ab 1078) were generous gifts of Nancy Rice (National Cancer Institute-Frederick Cancer Research and Development Center, Frederick, Md.) and were used for immunoblotting and Ab supershift assays.

Plasmids. To construct the pGST-RelA fusion plasmid, the *Sma*I-*Not*I RelA (p65) fragment containing full-length RelA cDNA derived from pCMVp65 (62) was inserted in frame into the pGEX-4T-2 vector (Pharmacia). To construct pGST-cRel, an *Xho*I-*Not*I fragment containing full-length c-Rel cDNA, derived from pBKCMV-cRel, was inserted in frame into the *Sal*I-*Not*I sites of the pGEX-4T-2 vector. The reporter plasmid 4xCD28RE-CAT (23) was constructed by inserting four copies of the CD28RE site of the IL-2 promoter into the multiple cloning site of pBLCAT2, a thymidine kinase (TK)-chloramphenicol acetyltransferase (CAT) reporter plasmid (51). The wild-type and CD28RE-mutated (sequence from −159 to −154 changed from 5′ AATTCC3′ to 5′CCTCGA3′ in the IL-2 promoter) IL-2–CAT reporter plasmids (12) were provided by Cornelis Verweij (University Hospital, The Netherlands). The expression plasmid pCMVp65 (62) was provided by Patrick Baeuerle (Ludwig Maximilians University, Martinsried, Germany), plasmid pCMVp50 (pCMV399 [33]) was provided by Alain Israël (Institut Pasteur, Paris, France), and plasmid pRSVcRel was described previously (73). Plasmid pRSV-IκBα (pRSV-MAD3) was constructed by inserting the *Hin*dIII-*Xba*I IκBα fragment derived from pMAD3 (29), a construct provided by Steven Haskill (University of North Carolina, Chapel Hill), into the pS-RSPA vector. The control expression vector pRc/CMV was purchased from Invitrogen, and the Rous sarcoma virus expression vector pS-RSPA (17) was a gift from David Derse (National Cancer Institute-Frederick Cancer Research and Development Center). Plasmids expressing activated Raf-1 (Raf-BXB) and an inactive mutant (BXB-301) were described previously (8).

Expression, purification, and cleavage of GST-RelA and GST-cRel fusion proteins. *Escherichia coli* DH5α was used as the expression host for both the glutathione *S*-transferase (GST)–RelA and GST-cRel fusion proteins. Crude bacterial extracts containing pGEX-4T-2, pGST-RelA, or GST-cRel were prepared as described by Kodonaga et al. (34). DH5α cells were grown overnight in 10 ml of LB medium containing 100 μg of ampicillin per ml. This culture was then mixed with 500 ml of LB medium containing 100 μg of ampicillin per ml and allowed to grow at 37°C. When the optical density reading at A_{550} was 0.1, isopropyl-β-D-thiogalactopyranoside (IPTG; Pharmacia) was added to 1 mM (final concentration). The continuous growth of bacteria was monitored until the optical density reached 0.4. Then the cells were centrifuged at 6,000 × *g* for 10 min and subsequently washed with 20 ml of LB medium. After centrifugation, the pellet was resuspended in 1.3 ml of buffer A (40 mM Tris-HCl [pH 7.8], 25% sucrose, 0.2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM dithiothreitol, 1 mM sodium metabisulfite); then 500 μl of buffer A containing 1 mg of lysozyme per ml was added, and the mixture was incubated at 4°C for 1 h. Following this, 1.2 ml of 10 M urea was added to the solution, which was left to incubate for 1 h. The mixture was then centrifuged at 63,000 × *g* for 1 h. The supernatant was dialyzed in 200 ml of buffer B (20 mM Tris-HCl [pH 7.8], 50 mM KCl, 10 mM $MgCl_2$, 10 μM $ZnSO_4$, 1 mM EDTA, 20% glycerol, 1 mM dithiothreitol, 0.2 mM phenylmethylsulfonyl fluoride, 1 mM sodium metabisulfite, 1 M urea) for 90 min, then in 500 ml of buffer B for 2 h, and finally in 500 ml of buffer B overnight. To purify the fusion proteins, bacterial lysates containing fusion proteins were incubated with GST-agarose beads (Pharmacia) for 1 h at 4°C. After an extensive wash with phosphate-buffered saline, the fusion proteins either were eluted from the beads or underwent further cleavage with thrombin for 2 h at room temperature. The protein concentration was measured by a protein assay kit (Bio-Rad), using the Lowry method (49).

Electrophoresis mobility shift assay (EMSA). The CD28RE within the IL-2 promoter was synthesized with a Cyclone Plus DNA synthesizer (Millipore) and used as the DNA probe. The DNA probe was radiolabeled with [^{32}P]dGTP by using the Klenow fragment of DNA polymerase I (Boehringer Mannheim). Competitor DNAs were obtained by annealing the synthetic oligonucleotides. For the binding reaction, 0.1 ng of the radiolabeled CD28RE probe (10,000 to 30,000 cpm) was incubated with 3 μg of nuclear extract prepared from human peripheral blood T cells. To test if the recombinant protein expressed in bacteria can bind to the oligonucleotide probe, 5 μg of bacterial lysates containing GST or GST-RelA fusion protein or 0.1 to 0.6 μg of purified recombinant GST-RelA, GST-cRel, or RelA was used in the binding reaction. The binding buffer contained 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM dithiothreitol, 5% glycerol, and 2 μg of poly(dI-dC). To test the binding of the purified recombinant protein, 1 μg of bovine serum albumin was added to the binding buffer and 500 ng instead of 2 μg of poly(dI-dC) was used. This 20 μl of the reaction mixture was left to incubate at room temperature for 20 min. If competitor DNAs or antibodies were added, they were preincubated for 10 min before addition of the radiolabeled probe. To examine protein dimerization, different recombinant proteins were preincubated together for 30 min at room temperature before addition of the radiolabeled probe. The final reaction mixture was analyzed on a 4% nondenaturing polyacrylamide gel with 0.25× Tris-borate-EDTA electrophoresis buffer. The radiolabeled probe and competitors used are described in Table 1.

DNA transfection and CAT assay. The Jurkat T-cell clone J.LEI or E6-1 (5 × 10^6 cells) was transfected transiently by electroporation (Bio-Rad) at 960 μF and 200 V as described previously (73). The cells were harvested 48 h after transfection and subsequently lysed by three freeze-thaw cycles. The protein concentrations were measured by a protein assay kit (Bio-Rad), using the Lowry method (49). CAT activities were quantitated by using CAT enzyme-linked immunosorbent assay (ELISA) assay kits (5 Prime→3 Prime, Inc.) or by a thin-layer chromatography (TLC) method (25). For the CAT ELISA, 40 μg of total cellular protein was mixed with dilution buffer to a total of 200 μl and placed into anti-CAT Ab-coated wells. The reaction mixture was incubated at room temperature for 2 h. The wells were then washed five times with washing buffer. Next, 200 μl of a solution containing a biotinylated anti-CAT Ab was added, and the mixture was incubated for 1 h. After washing with washing buffer, 200 μl of a solution containing streptavidin-conjugated alkaline phosphatase was added, and the mixture was incubated for 30 min at room temperature. The wells were washed again with washing buffer, a substrate (*p*-nitrophenyl phosphate) was added to each well, and the wells were left to incubate for 30 min at 37°C. CAT concentrations, expressed as picograms per 40 μg of protein, were measured by using an ELISA reader (SLT Spectra). Because the IL-2 promoter is weak, we measured the expression of the IL-2–CAT reporter by using the more sensitive TLC method (25). The TLC plate was exposed to a film for 2 to 5 days at −70°C with intensifying screens.

RESULTS

RelA (p65) is present in the CD28RC induced by CD28 costimulation. To investigate the role of RelA (p65) in the CD28 signaling pathway, we studied the CD28-induced DNA-binding factors that bound the CD28RE. Human peripheral blood T cells were treated with PMA, anti-CD28 MAb 9.3 (100 ng/ml), or PMA plus anti-CD28 MAb for 7 h. The nuclear extracts were then prepared and subjected to EMSA. The binding reaction and the addition of unlabeled oligonucleotide

TABLE 1. Oligonucleotides used

Name	Sequence
CD28RE	5′GATCGTTTAAAGAAATTCCAAA3′ CAAATTTCTTTAAGGTTTCTAG (−167 to −150 within the human IL-2 promoter)
CD28RE-mutant	5′GATCGTTTAAAGAAATTggAAA3′ CAAATTTCTTTAAccTTTCTAG
HIV-κB	5′GATCAAGGGACTTTCCGCTGGGGACTTTCCAG3′ TTCCCTGAAAGGCGACCCCTGAAAGGTCCTAG (−106 to −79 within the HIV long terminal repeat)
CD28RE-2	5′AGCTTGGGCCAGGAGATTCCACAACTCAGGTAGTT3′ ACCCGGTCCTCTAAGGTGTTGAGTCCATCAAGATC (−114 to −84 within the mouse GM-CSF promoter)
CD28RE-3	5′AGCTTAGGATGAGATTCCACTGCATAGAAAGT3′ ATCCTACTCTAAGGTGACGTATCTTTCAGATC (−204 to −177 within the mouse IL-3 promoter)
CD28RE-4	5′AGCTTCTATGGAGGTTCCATGTCAGATAAAGT3′ AGATACCTCCAAGGTACAGTCTATTTCAGATC (−119 to −92 within the mouse IL-3 promoter)
CD28RE-5	5′AGCTTGTCTAAAGGAAACTCTAACTACAACACCA3′ ACAGATTTCCTTTGAGATTGATGTTGTGGTTCGA (−170 to −140 within the human IFN-γ promoter)
CD28RE-5-mutant	5′AGCTTGTCTAAAGctgcagCTAACTACAACACCA3′ ACAGATTTCgacgtcGATTGATGTTGTGGTTCGA
NFAT	5′GAGGAAAAACTGTTTCATACAGAAGGCG3′ CTCCTTTTTGACAAAGTATGTCTTCCGC (−285 to −254 within the human IL-2 promoter)

competitors were performed as described in Materials and Methods. Treatment of human peripheral blood T cells with the anti-CD28 MAb plus PMA resulted in one specific complex binding to the CD28RE oligonucleotide probe (Fig. 1A, lane 4). This complex was present, but barely discernible, when nuclear extract from PMA-treated T cells was used in the binding mixture (lane 2), whereas anti-CD28 MAb treatment alone did not induce any DNA-protein complex (lane 3). However, longer treatment (40 h) of T cells with PMA alone induces detectable levels of CD28RC that binds to the CD28RE (10). The CD28RE oligonucleotide containing a mutation (see Table 1) was unable to compete for binding to the induced CD28RC (lane 6), which suggests that these nuclear factors bind specifically to the CD28RE oligonucleotide. To further confirm that the CD28RC induced in the PMA-plus-anti-CD28-stimulated T cells contained RelA (p65), antibody supershift experiments were performed with an antiserum (Ab 1226) against RelA (p65). The Ab was preincubated with the reaction mixture before addition of the radiolabeled CD28RE oligonucleotide. Addition of the antiserum against RelA (p65) resulted in a supershift accompanied by a diminished intensity in the induced complex (Fig. 1B, lanes 2 and 3). In contrast, two control Abs did not induce a supershifted band (lanes 4 and 5), which clearly indicates that RelA (p65), or an immunologically related protein, was contained in the CD28RC induced by PMA-plus-anti-CD28 stimulation.

Recombinant RelA (p65) can bind to the CD28RE of the IL-2 promoter as a homodimer. To investigate if RelA (p65) can bind directly to the CD28RE site in the IL-2 promoter, we constructed a GST-RelA fusion plasmid (pGST-RelA). This fusion plasmid was transfected into *E. coli*, and the transformant was stimulated with IPTG to induce fusion protein production. Western blot (immunoblot) analysis identified the expected 100-kDa GST-RelA fusion protein by the anti-RelA (p65) antiserum (Ab 1226) (37a). To test the binding of RelA (p65) to the CD28RE oligonucleotide, we performed an

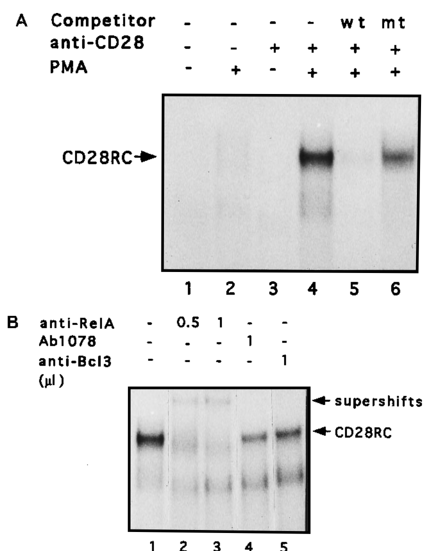

FIG. 1. RelA (p65) is present in the CD28RC induced by CD28 costimulation. (A) PMA-plus-anti-CD28 MAb stimulation induced the CD28RC. Nuclear extracts containing 3 μg of total protein from different stimuli were analyzed by EMSA. The ^{32}P-labeled oligonucleotide containing the CD28RE site of the IL-2 promoter was used as a probe. A competition study was done with an unlabeled CD28RE wild-type (wt) or mutant (mt) oligonucleotide. The sequences of competitors are shown in Table 1. The competitors were added 10 min before addition of the radiolabeled CD28RE oligonucleotide probe. After addition of the radiolabeled probe, the whole reaction mixture was incubated for 20 min and then analyzed on a 4% native polyacrylamide gel. (B) Anti-RelA (p65) antisera supershifted the CD28RC. Before analysis in EMSA, either 0.5 or 1 μl of anti-RelA (p65) Ab 1207 or two control Abs, Ab1078 and anti-Bcl-3, were added to the reaction mixture. The whole mixture was incubated for 10 min before addition of the labeled CD28RE probe. After addition of the labeled CD28RE, the reaction mixture was incubated for an additional 20 min. The reaction mixture was then analyzed on a 4% polyacrylamide gel. Both the induced CD28RC and the supershifted bands are shown by arrows.

EMSA analysis. A protein-DNA complex was detected after incubation of the radiolabeled CD28RE oligonucleotide with bacterial lysate containing the GST-RelA fusion protein (Fig. 2A, lane 3). No evidence of a complex that bound the CD28RE oligonucleotide probe was found in the bacterial lysate containing GST (lane 2). Addition of unlabeled CD28RE oligonucleotide or human immunodeficiency virus (HIV) κB (HIV-κB) oligonucleotide (indicated as NFκB in Fig. 2B) in 25-, 50-, or 100-fold molar excess inhibited the binding of RelA (p65) to the radiolabeled CD28RE oligonucleotide (Fig. 2B, lanes 2 to 4 and 8 to 10). The unlabeled CD28RE oligonucleotide containing two mutated residues (TTCC was changed to TTgg) did not compete with the labeled wild-type CD28RE probe (lanes 5 to 7). This finding demonstrates the binding specificity of RelA (p65) to the CD28RE; i.e., RelA (p65) bound to the wild-type but not the mutant CD28RE. To further confirm that the DNA-protein complex contained RelA (p65), we conducted Ab supershift assays with the antiserum against RelA (p65). As expected, the antiserum against RelA (p65) (Fig. 2C, lanes 4 and 5), but not the control antiserum against c-Rel (lane 6), supershifted the DNA-protein complex. To examine whether RelA (p65) bound to the CD28RE as a monomer or a homodimer, GST-RelA was purified from whole bacterial lysate by using GST-agarose beads. The recombinant RelA (p65) was prepared from cleavage of the GST-RelA with thrombin. In Fig. 2D, recombinant RelA (p65) bound to the CD28RE, as shown by the faint band (lane 6). A combination of GST-RelA and RelA (p65) resulted in a new band positioned between GST-RelA and RelA (p65) by using either the

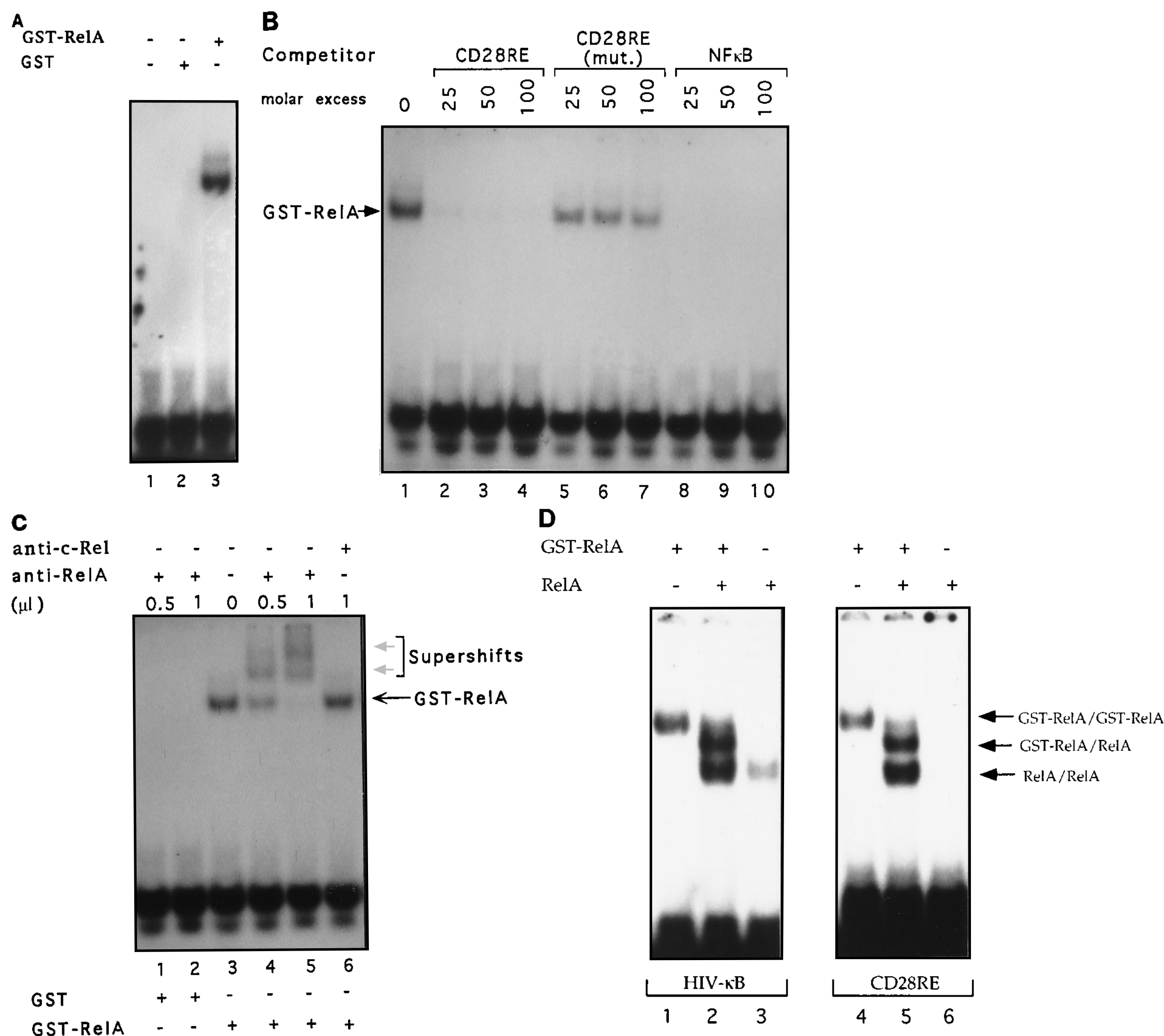

FIG. 2. Recombinant RelA (p65) binds to the CD28RE site within the IL-2 promoter. (A) Recombinant GST-RelA induced a shifted DNA-protein complex in EMSA analysis. Five micrograms of the bacterial lysate from either pGST-RelA- or pGEX-4T-2-transformed *E. coli* was incubated with the ^{32}P-labeled CD28RE oligonucleotide probe and analyzed on a 4% polyacrylamide gel. (B) Wild-type CD28RE and HIV-κB (indicated as NFκB) oligonucleotides but not the CD28RE mutant (mut.) competed with GST-RelA binding to the radiolabeled CD28RE probe. A total of 5 μg of pGST-RelA-transfected bacterial lysate was included in the mixture with or without competitors. The competitors were added 10 min before addition of the radiolabeled CD28RE probe; the amount of each competitor added is indicated by a molar ratio. After addition of the radiolabeled probe, the reaction mixture was analyzed on a 4% polyacrylamide gel. (C) Antisera against RelA (p65) supershifted the DNA-protein complex. For the supershift assay, either 0.5 or 1 μl of anti-RelA (p65) or 1 μl of anti-c-Rel antiserum was added into the reaction mixture 10 min before addition of the radiolabeled CD28RE probe. After addition of the radiolabeled CD28RE probe, the final reaction mixture was analyzed on a 4% polyacrylamide gel. The supershifted bands are indicated. (D) RelA (p65) bound to the CD28RE as a homodimer. To test whether the RelA (p65) homodimer bound to the CD28RE, GST-RelA was purified from whole bacterial lysate. Recombinant RelA was obtained by cleavage of the GST-RelA with thrombin. GST-RelA (0.6 μg) and RelA (0.3 μg) were preincubated for 30 min at room temperature before addition of the radiolabeled probe (either the HIV-κB or the CD28RE probe). The final reaction mixture was analyzed on a 4% polyacrylamide gel.

HIV-κB or the CD28RE probe (lanes 2 and 5). This finding suggests that as for the binding to the HIV-κB site, RelA (p65) bound to the CD28RE as a homodimer. Because the CD28RE site corresponds to a region within the IL-2 promoter involved in T-cell activation, the data shown above indicate that RelA (p65) may be a necessary and critical transcription factor responsible for activation of the CD28RE-containing genes.

RelA (p65) can also bind to other, similar CD28RE motifs in the GM-CSF, IL-3, and IFN-γ promoters. CD28 costimulation leads to up-regulation of mRNAs of many lymphokines, such as tumor necrosis factor alpha, GM-CSF, IL-1, IL-2, IL-3, IL-4, IL-5, and IFN-γ (11, 26, 45, 79), and it has been proposed that many lymphokine genes may possess a similar CD28RE motif (21). To assess whether GST-RelA can also bind to similar CD28RE motifs within the GM-CSF, IL-3, and IFN-γ promoters, we performed EMSA using double-stranded oligonucleotides corresponding to (i) nucleotides −114 to −84 of the mouse GM-CSF 5′ region (CD28RE-2), (ii) nucleotides −204 to −177 (CD28RE-3) of the mouse IL-3 5′ region, (iii) nucleotides −119 to −92 (CD28RE-4) of the mouse IL-3 5′ region, and (iv) nucleotides −170 to −140 (CD28RE-5) of the human IFN-γ 5′ region as unlabeled competitors. Competitors with a 100-fold molar excess of unlabeled oligonucleotides of similar CD28RE motifs in GM-CSF or IL-3 specifically inhibited the

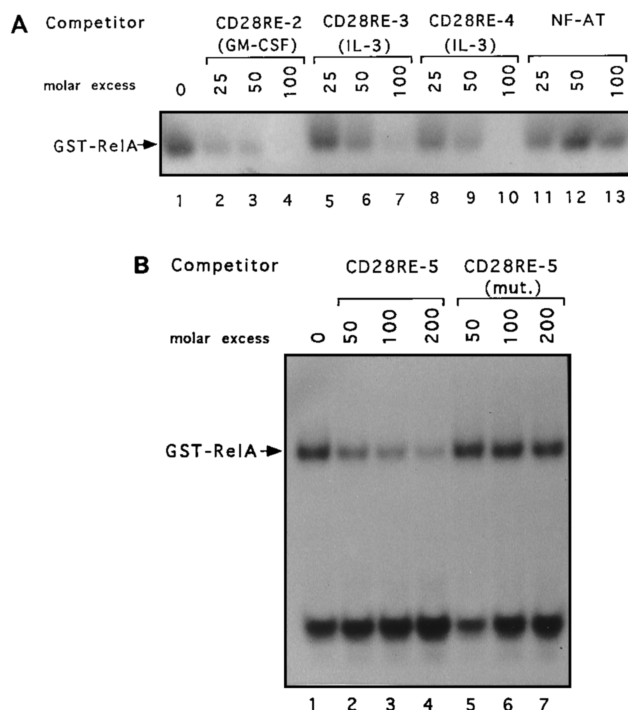

FIG. 3. GST-RelA binds to the conserved CD28RE motifs within the GM-CSF, IL-3, and IFN-γ promoters. (A) GST-RelA bound to the conserved CD28RE motifs in GM-CSF and IL-3 promoters. EMSA analysis and the addition of competitors were performed as described in the text. The sequences of the competitors are shown in Table 1. (B) GST-RelA bound to the conserved CD28RE motif within the IFN-γ promoter. An oligonucleotide encompassing positions −170 to −140 from the transcription initiation site of the IFN-γ promoter was used as a competitor. The mutant (mut.) was generated by changing six nucleotides of the wild-type sequence as shown in Materials and Methods.

binding of GST-RelA with the radiolabeled CD28RE probe (Fig. 3A, lanes 4, 7, and 10). The unlabeled NFAT oligonucleotide, representing a unique site within the IL-2 promoter, did not show any significant competition (lanes 11 to 13). Competition was also evident in the unlabeled oligonucleotide containing the conserved CD28RE motif within the IFN-γ promoter (Fig. 3B, lanes 2 to 4). These results further suggest that CD28 costimulation leads to the regulation of many cytokine genes, probably through RelA (p65) and possibly other Rel family members. In addition, on the basis of comparison of these conserved CD28RE sequences, we propose that the CD28RE consensus sequence (RGARNTTCC) is a RelA (p65)-binding motif (Table 2).

RelA (p65) activates transcription of the CD28RE motif of the IL-2 promoter and is inhibited by IκBα. Direct binding of RelA (p65) to the CD28RE implicates its potential role as a direct regulator of the CD28RE enhancer activity in vivo. The potential effect of RelA (p65) on the CD28RE enhancer was examined. Four copies of CD28RE sites of the IL-2 promoter linked to the CAT reporter gene were generated as a reporter construct (4xCD28RE-CAT [23]). The RelA (p65) expression vector pCMVp65 and the empty vector pRc/CMV, alone or in combination, were used in cotransfection assays with the 4xCD28RE-CAT reporter gene. Increasing amounts of the pCMVp65 expression vector were tested to determine its potential transcriptional activity on the reporter gene. The total amounts of plasmids added were normalized by using the empty vector pRc/CMV. As shown in Fig. 4, transfection of the RelA (p65) expression vector resulted in a very potent activation of CAT activity. When 10 μg of pCMVp65 was cotransfected with the reporter plasmid, up to a 55-fold induction was detected compared with the level found after cotransfection with the empty vector alone. The effect of RelA (p65) on CD28RE-CAT was not through the TATA box, since 10 μg of pCMVp65 did not activate the control TK-CAT reporter gene, encoded by pBLCAT2 (Fig. 4). One of the mechanisms to regulate the activation by NF-κB is through a cytoplasmic inhibitor, IκBα, which retains NF-κB proteins in the cytosol (60). To determine if IκBα can inhibit the functional activity of RelA (p65) on CD28RE-CAT, Jurkat T cells were transfected with the 4xCD28RE-CAT reporter plasmid and pCMVp65, pRSV-IκBα, or pCMVp65 plus pRSV-IκBα. Although RelA (p65) activated CD28RE-driven transcription of 4×CD28RE-CAT, coexpression of IκBα significantly inhibited its activity (Fig. 4). Overexpression of IκBα alone did not show any effect on the 4xCD28RE-CAT reporter.

TABLE 2. Conserved CD28REs in the 5′ flanking regions of lymphokine genes

Gene	Region[a]	Sequence[b]
IL-2		
Human	−161 to −153	AGAAATTCC
Mouse	−163 to −155	AGAAATTCC
IL-3		
Human	−118 to −110	GGAGGTTCC
Mouse	−114 to −106	GGAGGTTCC
	−199 to −191	tGAGATTCC
Human IL-8	−80 to −72	GGAATTTCC
IFN-γ		
Human	−154 to −162	AGAGTTTCC
Mouse	−161 to −169	AGAGTTTCC
Human IFN-β	−64 to −56	GGAAATTCC
GM-CSF		
Human	−95 to −87	GGAGATTCC
Mouse	−107 to −99	GGAGATTCC
G-CSF		
Human	−187 to −179	AGAGATTCC
Mouse	−191 to −183	AGAGATTCC
Consensus sequence		R<u>GA</u>RN<u>TTCC</u>

[a] Numbers represent relative distances from the transcription start site.
[b] R = A or G; N = A, T, C, or G. The base that is different from the consensus sequence is lowercase. Bases underlined are 100% conserved.

The RelA (p65)–c-Rel heterodimer can bind to the CD28RE and synergistically activate its enhancer activity. c-Rel itself was shown to bind to the CD28RE (23). After demonstrating that RelA (p65) also binds to the CD28RE, we studied if the heterodimer of these two proteins binds to the CD28RE. If it does, what is the functional significance of this heterodimer? To test this possibility, we constructed GST–c-Rel and prepared the purified recombinant c-Rel protein. Consistent with our previous report (23), both GST–c-Rel and c-Rel bound to the CD28RE probe (Fig. 5A, lane 4) (37a). Compared with the GST–c-Rel–DNA complex, which moved much more slowly, RelA (p65) and c-Rel protein-DNA complexes showed similar gel mobilities in an EMSA analysis (37a). To distinguish the mobility shift upon heterodimer formation, we used GST–c-Rel instead of the recombinant c-Rel. Similar to the result for the HIV-κB probe, the addition of RelA (p65) incrementally resulted in the formation of a new band at a position between GST–c-Rel and RelA (p65) homodimers (Fig. 5A, lanes 2 and 5 to 7). This finding suggests that GST–c-Rel and RelA (p65) heterodimers bound to both the HIV-κB and CD28RE elements. We then examined the possible functional interactions

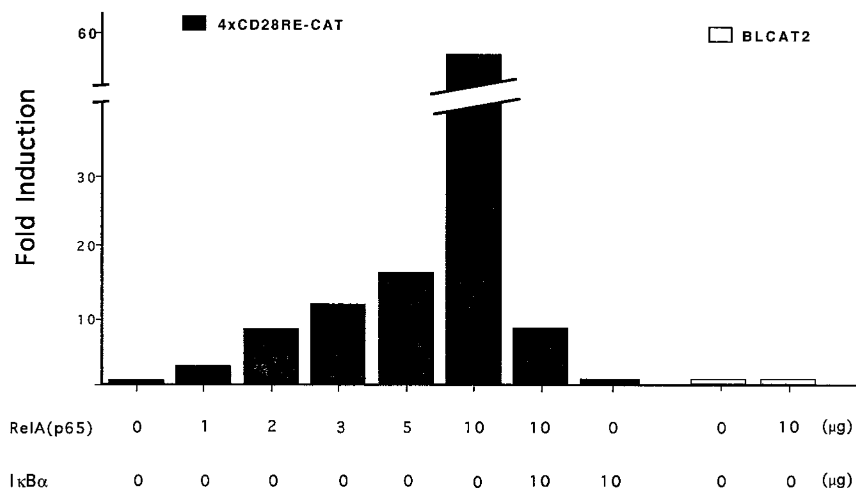

FIG. 4. Dose-dependent, functional activation of the CD28RE by RelA (p65), which is inhibited by overexpression of IκBα. Jurkat T cells (clone J.LEI or E6-1) (5×10^6) were transfected with 10 μg of the 4xCD28RE-CAT reporter plasmid and 10 or 20 μg of effector plasmid by electroporation. To show dose-dependent responses, different amounts of pCMVp65 were transfected with or without the empty vector (pRc/CMV) to make 10 μg of total effector plasmid. To determine whether this effect can be inhibited by overexpression of IκBα, a total of 20 μg of effector plasmids was transfected into Jurkat T cells. Empty vector (pS-RSPA) was added to normalize the amount of transfected effector plasmid. For another control, 10 μg of the TK-CAT plasmid pBLCAT2 was used as a reporter gene. CAT concentrations were measured 48 h after electroporation and expressed as picograms per 40 μg of cellular extract. For comparison, the final results are represented as fold induction (averages of three independent transfection experiments).

between these two proteins. A fixed amount of pCMVp65 (3 μg) was cotransfected with various amounts of pRSVcRel (3, 10, and 20 μg) and with 10 μg of the 4xCD28RE-CAT reporter. The total amount of transfected plasmids for each sample was normalized by adding an empty vector, pRc/CMV or pS-RSPA. Jurkat T cells were harvested 48 h after transfection, and the CAT concentrations were determined as described in Materials and Methods. Transfection with 10 μg of pRSVcRel activated the 4xCD28RE-CAT reporter approximately twofold (Fig. 5B), less than in our previous study (23). The discrepancy between these two results is likely due to the lower amount (10 μg) of the reporter plasmid used in this study (20 μg was used in our previous study). Furthermore, different Jurkat clones (established at the National Cancer Institute-Frederick Cancer Research and Development Center and Baylor College of Medicine) were used in these two studies. Consistently, cotransfection of c-Rel and RelA (p65) activated the CD28RE synergistically, and this synergism was dose dependent (Fig. 5B). To verify that the synergistic effect of c-Rel and RelA (p65) was mediated through the CD28RE site directly, the same experiments were performed by using the enhancerless TK-CAT reporter pBLCAT2. In contrast to the results for the CD28RE-CAT reporter, there was no synergism between RelA (p65) and c-Rel when pBLCAT2 was used as the reporter (Fig. 5C). Given the synergistic effect of these two proteins on activating the CD28RE in vivo, the heterodimeric form may be a more potent activator than either of the homodimeric forms.

Activated Raf-1 kinase synergizes with RelA (p65) in activating the CD28RE enhancer activity. c-*raf-1*, the cellular homolog of the v-*raf* transforming gene, encodes a serine/threonine kinase that is activated after stimulation of cell surface receptors such as growth factor receptors and cytokine receptors (2, 59, 76). In resting T cells, it has been shown that anti-CD3 treatment increases the expression of c-*raf-1* mRNA approximately fourfold and induces its kinase activity (84). IL-2 treatment of T cells further up-regulates Raf-1 expression and its kinase activity (84). Stimulation with an Ab against CD2 or CD28 molecules on T cells also induces Raf-1 kinase activity (67). However, the regulation of the Raf-1 kinase through IL-2 and T-cell receptor signaling is quite distinct in that IL-2 stimulation enhances phosphorylation on tyrosine residues and T-cell receptor stimulation results in phosphorylation on serine residues of the Raf-1 kinase (68, 76). By cotransfection assays, activated Raf-1 kinase has been shown to increase transcription of the IL-2 promoter stimulated with anti-CD3 plus PMA or phytohemagglutinin plus PMA (56). Significantly, inactive Raf-1 mutant inhibits the induced transcription of the IL-2 promoter (56). It has been shown that the activated Raf-1 kinase can mediate its activity through the serum response element-, AP-1-, and Ets-binding motifs (8, 82). We and others have recently identified the κB sites in HIV long terminal repeat as the response element for Raf-1 kinase activity (9, 19). Since AP-1, κB, and CD28RE sites are present within the IL-2 promoter, we were interested in determining whether Raf-1 kinase can activate the CD28RE enhancer activity. Raf-BXB, an N-terminally truncated Raf-1 shown to be constitutively active, or its inactive mutant BXB-301 was cotransfected with the CD28RE-CAT reporter gene. In contrast to the activation of the κB enhancer as demonstrated previously (9, 19, 44), the activated Raf-1 kinase itself did not activate the CD28RE (Fig. 6). Surprisingly, cotransfection of Raf-BXB with RelA (p65) activated the CD28RE up to 14-fold, compared with 3-fold by RelA (p65) alone; this synergism was not evident in BXB-301 mutant and RelA (p65) cotransfection. This result suggests that Raf kinase may mediate its effect in part by enhancing the transcriptional activation of the CD28RE within the IL-2 promoter.

RelA (p65) synergizes with an anti-CD28 MAb in activating the CD28RE enhancer activity. CD28 ligation alone cannot induce any significant changes in T cells. However, Åsjö et al. (4) reported that CD28 ligation alone causes replication of HIV type 1 (HIV-1) in some HIV-1-infected T cells. To examine whether overexpression of RelA (p65) could potentiate the CD28 signaling effect, we tested the possible synergism between RelA (p65) and anti-CD28 stimulations. Jurkat T cells were cotransfected with a suboptimal dose (3 μg) of expression vector pCMVp65 and 10 μg of reporter plasmid

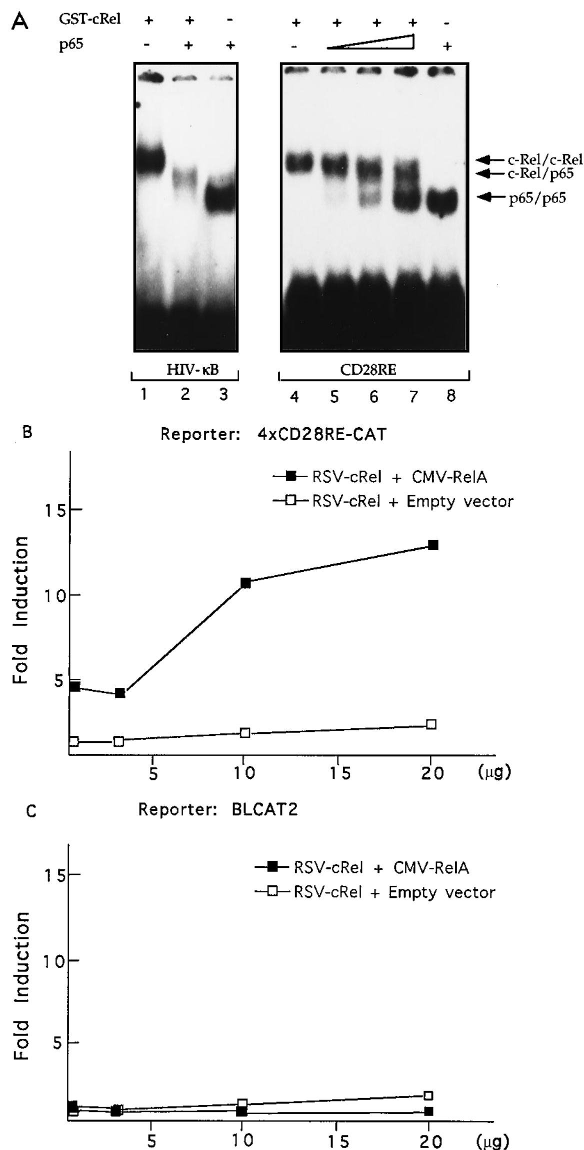

FIG. 5. RelA–c-Rel heterodimer can bind to the CD28RE and activate the CD28RE synergistically. (A) RelA (p65) can bind to the CD28RE as a heterodimer with c-Rel. Purified GST–c-Rel (0.6 μg) was preincubated with RelA (p65) (0.1, 0.3, or 0.6 μg) for 30 min at room temperature before addition of the radiolabeled probes. Either the HIV-κB or the CD28RE probe was used to study binding of the RelA–c-Rel heterodimer. The final reaction mixture was analyzed on a 4% polyacrylamide gel. The homo- and heterodimeric forms are indicated by arrows. (B) RelA (p65) and c-Rel activate the CD28RE synergistically. Jurkat T cells were cotransfected with various amounts (3, 10, and 20 μg) of pRSVcRel and 10 μg of the 4xCD28RE-CAT (B) or pBLCAT2 (C) reporter plasmid in the presence or absence of 3 μg of the pCMVp65 expression vector. The amount of transfected plasmids was normalized by adding the empty vector pS-RSPA or pRc/CMV. The cells were harvested 48 h after transfection. CAT concentrations were determined and compared with that of the empty vector, and the results are expressed as fold induction (averages of three independent transfection experiments).

4xCD28RE. Cells were then treated with or without anti-CD28 ascites at a dilution of 1:2,000 24 h after transfection. Cells were harvested after an additional 24-h incubation period. A combination of anti-CD28 MAb with RelA (p65) synergistically activated the CD28RE-CAT reporter gene (Fig. 7). The anti-CD28 MAb alone did not activate CD28RE-CAT expression. The synergism was not detected when CD28RE-CAT was

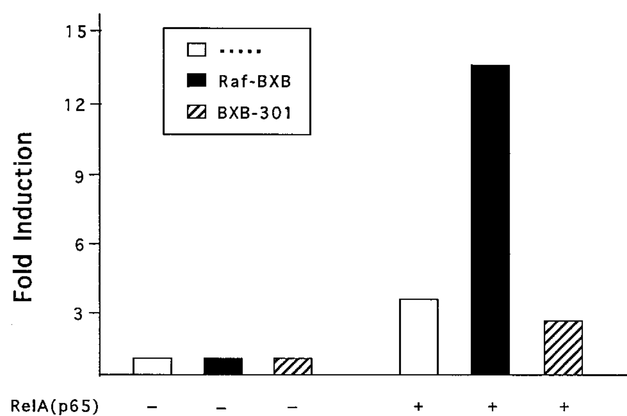

FIG. 6. Activated Raf-1 kinase enhances the transcriptional activation of CD28RE by RelA (p65). The empty vector pRc/CMV or the expression vector pCMVp65 (3 μg) with or without Raf-BXB (10 μg) or BXB-301 (10 μg) was cotransfected with reporter plasmid CD28RE-CAT (10 μg) into Jurkat T cells. The amount of transfected plasmids was normalized by adding the empty vector pS-RSPA or pRc/CMV. The cells were harvested 48 h after transfection, and CAT concentrations were measured. The results are presented as averages of fold induction.

replaced with the enhancerless TK-CAT reporter plasmid pBLCAT2.

RelA (p65) activates the enhancer activity of the wild-type IL-2 promoter but not the CD28RE-mutated IL-2 promoter. To examine if the transcriptional activity of RelA (p65) on the CD28RE is reproducible on the wild-type IL-2 promoter, both the wild-type and CD28RE-mutated IL-2–CAT reporter constructs, IL2-CAT and IL2(ΔCD28RE)-CAT respectively, were used. IL2(ΔCD28RE)-CAT contains the sequence 5′CCTC GA3′ in place of 5′AATTCC3′ at positions −159 to −154 of the IL-2 promoter (Fig. 8A and reference 12). The wild-type and mutant IL-2–CAT constructs were either transfected alone or cotransfected with the empty vector or the RelA (p65) expression vector. CAT activity was determined 48 h after cotransfection. As shown in Fig. 8B, cotransfection with the RelA (p65) expression vector further activated the wild-type IL-2–CAT construct but not the IL2(ΔCD28RE)-CAT con-

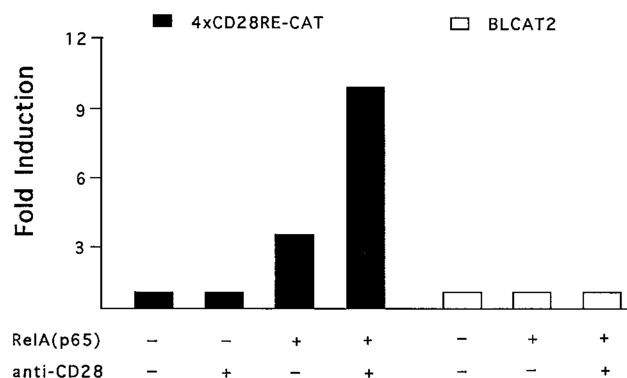

FIG. 7. Anti-CD28 MAb synergizes with RelA (p65) in activating the CD28RE. Three micrograms of the effector plasmid pCMVp65 or the empty vector pRc/CMV was cotransfected with 10 μg of the reporter plasmid 4xCD28RE-CAT into Jurkat T cells by electroporation. For the control experiment, 10 μg of TK-CAT reporter plasmid pBLCAT2 was used instead. For CD28 stimulation, anti-CD28 MAb ascites (clone 9.3; dilution of 1:2,000) was added to the medium 24 h after transfection. After incubation for an additional 24 h, cells were harvested and CAT concentrations were measured. The averages of fold induction are presented.

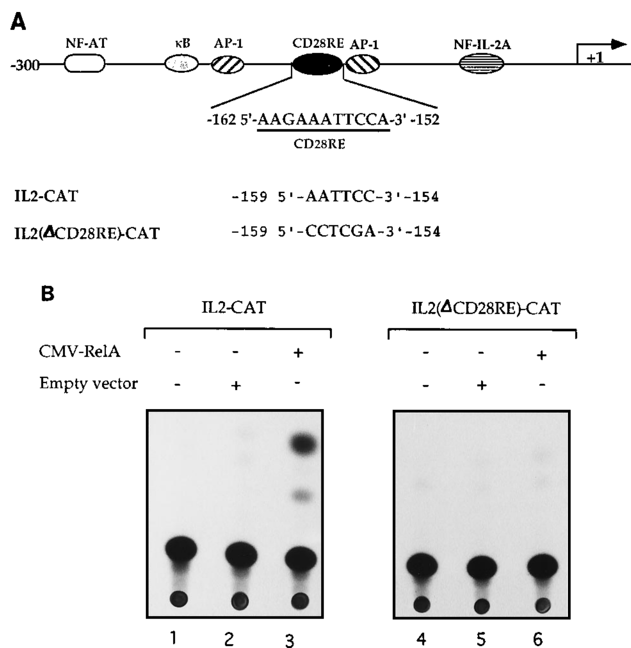

FIG. 8. RelA (p65) activates the wild-type but not the CD28RE-mutated IL-2 promoter. (A) Diagram of the IL-2 promoter with known protein-binding elements. The nucleotide sequence changes in the IL2(ΔCD28RE)-CAT construct are indicated (12). (B) RelA (p65) activates the wild-type but not the CD28RE-mutated IL-2 promoter. Ten micrograms of effector plasmid pCMV-p65 (CMV-RelA) or pCMV (empty vector) was cotransfected with 3 μg of either the CD28RE-mutated or wild-type IL-2 promoter reporter construct into Jurkat T cells. The basal activities of the reporter constructs without cotransfection of expression vectors were also examined. The cells were harvested 48 h after transfection, and CAT activity was measured as described in Materials and Methods. Results of one representative experiment of three independent experiments with identical results are shown.

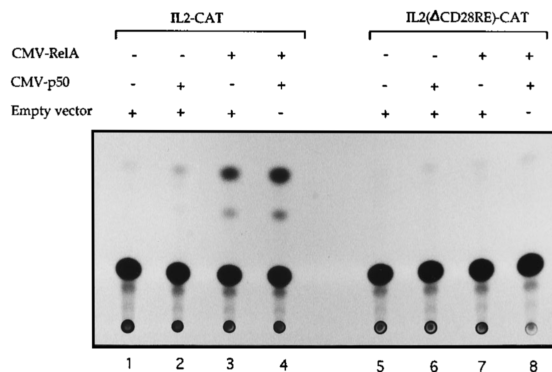

FIG. 9. RelA (p65)–NFKB1 (p50) heterodimer activates the IL-2 promoter via the CD28RE site. Ten micrograms of pCMV-p65 (CMV-RelA) or pCMV-p50 (CMV-p50), alone or in combination, was cotransfected with 3 μg of either the CD28RE-mutated or wild-type IL-2 promoter reporter construct into Jurkat T cells. pRcCMV (empty vector) was used to normalize the amounts of the transfected plasmids. The conversion of [^{14}C]chloramphenicol to its acetylated forms was measured by using a Betagen Betascope. Fold inductions compared with that with the empty vector alone were 1, 1.49, 5.34, and 9.30 (lanes 1 to 4) and 1, 1.16, 1.37, and 1.41 (lanes 5 to 8).

struct compared with cotransfection with the empty vector alone (compare lanes 3 and 2 and lanes 6 and 5). This result further demonstrates that the activation of RelA (p65) on the isolated CD28RE element was reproducible on the wild-type IL-2 promoter.

The RelA (p65)–NFKB1 (p50) heterodimer activates the IL-2 promoter via the CD28RE. We and others have determined that CD28, in addition to RelA (p65) and c-Rel, costimulation enhances nuclear translocation of NFKB1 (p50) (10, 13). The nuclear translocated NFKB1 (p50) is part of the complex that binds to the CD28RE (23). However, overexpression of NFKB1 (p50) inhibited the effect of RelA (p65) on the 4xCD28RE-CAT reporter construct (37a). Although overexpression of NFKB1 (p50) is suggested to play an inhibitory role in the regulation of some other genes (32, 35, 55), the depletion of NFKB1 (p50) results in the suppression of CD28 costimulatory events (13, 64). The proliferation of human peripheral blood T cells by anti-CD2-plus-anti-CD28 treatment is inhibited by incubation with NFKB1 (p50) antisense oligonucleotides (13). The T-cell proliferation induced by T-cell receptor and anti-CD28 costimulation is also significantly reduced in NFKB1 (p50)-deficient mice (unpublished results cited in reference 64). These inconsistent roles of NFKB1 (p50) led us to reexamine its role in regulation of the CD28RE in the context of the wild-type IL-2 promoter. As shown in Fig. 9, transfection with NFKB1 (p50) alone did not significantly activate wild-type IL-2 promoter activity. In contrast, cotransfection with RelA (p65) and NFKB1 (p50) resulted in a significant induction of the IL-2 promoter activity that was greater than that induced by RelA (p65) alone. Interestingly, this effect was not demonstrated by using the CD28RE-mutated IL-2 promoter reporter construct. This result suggests that the RelA (p65)–NFKB1 (p50) heterodimer activates the IL-2 promoter through the CD28RE.

DISCUSSION

In this study, we demonstrate that RelA (p65) not only is in the CD28RC but also is a strong transcriptional activator of the CD28RE in vivo. Direct binding of RelA (p65) to the CD28RE oligonucleotide suggests that RelA (p65) also binds to the non-κB element. Interestingly, this non-κB element (CD28RE) is not recognized by NFKB1 (p50) homodimers (23). RelA (p65) binds preferentially to the 3′ half-site of the κB sequences (36, 78). In support of this finding, mutations of the TTCC nucleotides at the 3′ end of the IL-2 CD28RE sequence prevented RelA (p65) from binding. Using random oligonucleotides generated by PCR, Kunsch et al. (36) identified a conserved sequence, GGGRNTTTCC, which is optimal for RelA (p65) binding. However, this high-affinity RelA-binding sequence motif does not necessarily include sequences with lower binding affinity which may also occur in vivo. On the basis of comparison of the conserved CD28RE motifs within different lymphokine promoters, we propose that a CD28 consensus sequence, RGARNTTCC, can also be bound by RelA (p65) (Table 2). Interestingly, a κB-like element (GGAGTT TCCT) in the tissue factor promoter, which is also bound by RelA (p65) alone, is compatible with the CD28RE consensus sequence (55). This CD28RE consensus sequence is distinct from the κB motif and the previously identified optimal RelA (p65)-binding motif (Table 3).

Activation of the IL-2 CD28RE-CAT reporter gene by RelA (p65) in vivo supports its critical role in CD28 signal transduction. Several recent reports indicate that RelA (p65) itself can form a homodimer and may activate the κB sites in the absence of NFKB1 (p50) (22, 36). Our results indicate the following: (i) the anti-RelA (p65) Ab supershifted the CD28RE DNA-protein complex which was induced by CD28 costimulation, (ii) RelA (p65) bound to the CD28RE directly in vitro, and (iii) the overexpression of RelA (p65) activated the CD28RE in a dose-dependent manner in vivo. These results strongly suggest

TABLE 3. Comparison of consensus and optimal binding sequences for Rel/NF-κB

Site[a]	Sequence[b]	Binding activity[c]			Reference(s)
		RelA	c-Rel	p50	
CD28RE	−RGARNTTCC	+	+	−	23, this report
κB	GGGRNNYYCC	+	+	+	6
Optimal RelA binding	GGGRNTTTCC	+	+	±	36
Optimal c-Rel binding	NGGNNWTTCC	±	+	±	36
Optimal p50 binding	GGGGATYCCC	±	+	+	36

[a] Optimal binding sequences were generated by PCR; no functional data are available yet.
[b] R, A or G; Y, C or T; W, A or T; N, A, T, C, or G.
[c] ±, variable binding (some bind and some do not bind), depending on the tested sequences.

that RelA (p65) is a potent regulator of genes containing the CD28RE by binding directly to the CD28RE. It was shown that CD28 costimulation results in further induction of reporter gene activation in Jurkat T cells transfected with IL-2, GM-CSF, IL-3, or IFN-γ promoter constructs (21). The binding of RelA (p65) to the CD28RE sites of these lymphokine promoters suggests the importance of RelA (p65) in regulating the expression of these lymphokine genes.

The synergism observed when RelA (p65) and c-Rel were cotransfected simultaneously provides further evidence of the different effects exerted by a combination of the Rel/NF-κB family proteins. In support of our results, the RelA (p65)–c-Rel complex induces very potent transcriptional activation in HeLa cells compared with the classical p65-p50 complex on κB-like elements in the human urokinase promoter region (27). The evidence that recombinant RelA (p65) and c-Rel form a heterodimer which binds to the CD28RE may support the synergistic effect found in vivo. In this regard, the different kinetics of the nuclear translocation of Rel/NF-κB family proteins induced by CD28 costimulation (10) may suggest that a transcription factor complex with lower activity can be substituted by a complex of higher activity in binding to the promoter region. The final outcome of transcriptional activation will depend on the combined effects of various amounts of transcription factors, the combination of transcription factors, the transcriptional activities of these individual factors, and their binding affinities toward the target genes.

The Raf-1 kinase is shown to work downstream of the Ras signaling pathway and upstream of MEK and mitogen-activated protein kinase pathways (for a review, see reference 83). Previously, we (9) and others (19, 44) showed that the activated Raf-1 kinase alone is sufficient to induce κB enhancer activity. The failure of the Raf-1 kinase alone to activate the CD28RE further suggests a difference in regulation between the κB site and the CD28RE. Our result also suggests that the effect of Raf kinase in T-cell activation is at least in part through the CD28RE within the IL-2 promoter. Raf-1 kinase may activate RelA (p65) through direct phosphorylation of RelA (p65) or may activate RelA-associated transcription factors which in turn synergize with RelA (p65) in induction of IL-2 gene expression.

In general, resting T cells do not proliferate after anti-CD28 stimulation alone. The signal delivered by CD28 stimulation is shown only in combination with potential initiation signals for cell activation. The initiation of these signals may be provided by treatment with a mitogen such as PMA and phytohemagglutinin or Abs against other cell surface molecules, such as CD2, CD3, or CD5 (47, 81). The activity of anti-CD28 stimulation is probably through amplification or modification of a preexisting signaling pathway. However, combined stimulation with mitogens or Abs against surface molecules may complicate the interpretation of CD28 signaling events. It has been shown that anti-CD28 costimulation may lead to tyrosine phosphorylation of the cytoplasmic tail of the CD28 receptor. This tyrosine-phosphorylated domain will then bind to phosphatidylinositol (PI) 3-kinase (57, 58, 70, 75). The PI 3-kinase does not bind to the unphosphorylated CD28 receptor; therefore, it is suggested that the CD28 signal transduction may involve the downstream signal activated by PI 3-kinase. However, the signal provided by anti-CD3 stimulation can also activate PI 3-kinase. Distinction between the downstream signals caused by different PI 3-kinase activators becomes difficult and complicates the dissection of the CD28 signaling events. Alternatively, secondary cross-linking of CD28 receptors (i.e., super-cross-linking by a second-step anti-immunoglobulin Ab treatment as opposed to cross-linking by the soluble bivalent Ab) provides a tool for studying CD28 signal transduction because this treatment results in T-cell activation. The signaling events induced by this treatment are not dependent on other stimuli such as PMA and anti-CD3 MAb. This treatment, however, does induce some effects that may not occur in vivo (54). For example, this treatment can induce increases in intracellular calcium levels; however, this effect is never found when PMA and soluble bivalent anti-CD28 MAb are used as the stimuli (39, 50).

The combination of suboptimal RelA (p65) levels and soluble anti-CD28 MAb, in the absence of PMA or other stimuli, in a cotransfection assay provides a very useful model system for the study of CD28 signal transduction. PMA stimulation of T cells can enhance nuclear translocation of RelA (p65) (10), and this effect is presumably replaced by that of the overexpressed RelA (p65). Thus far, the signal delivered from anti-CD28 stimulation in this combination remains unknown. Possibilities include a signal to functionally activate the RelA (p65) protein through phosphorylation or to activate other transcription factors which cooperate with RelA (p65) in gene activation. Our transfection results predict that in certain stages of T cells, in which RelA (p65) is highly expressed, CD28 signaling alone may be sufficient to induce T-cell activation. This may explain how CD28 signaling alone can induce HIV-1 replication in some HIV-1-infected T cells (4).

There are two motifs within the IL-2 promoter which have been shown to bind to the recombinant Rel/NF-κB transcription factors: the κB site and the CD28RE (references 23 and 40 and this report). The functional role of the κB motif in the IL-2 promoter is unclear (reviewed in reference 14). The activity of a CAT reporter construct containing an insertion of five copies of this κB motif is strongly induced by mitogens in El4 cells (63). However, deletion of this κB motif does not affect the transcriptional activity of the IL-2 promoter by PMA-plus-phytohemagglutinin stimulation (18). In addition, Jain et al. (30) showed that cross-linked anti-CD3ε MAb in-

duces the enhancer activity of several κB-mutated IL-2 promoter constructs to a degree comparable to or higher than that in the wild-type IL-2 promoter in T-cell clones. The difference among these results suggests the functional disparity of an enhancer either in a minimal promoter construct or in the context of the wild-type promoter. Interestingly, the element within the IL-2 promoter responsible for the activity of human T-cell leukemia virus type 1 Tax, shown to activate the κB site of the IL-2Rα promoter (60a), is located in the CD28RE but not in the κB site (43, 52). These studies further demonstrate the functional importance of the CD28RE within the IL-2 promoter in vivo. Our result suggests that neither RelA (p65) itself nor a combination of RelA (p65) and NFKB1 (p50) has any detectable functional activity on the NF-κB site of the IL-2 promoter in the absence of the CD28RE site. However, either RelA (p65) or a combination of RelA (p65) and NFKB1 (p50) activated the wild-type IL-2 promoter. This finding indicates that the RelA (p65) homodimer as well as the p65-p50 heterodimer activates the IL-2 promoter in a CD28RE-dependent manner. The proximal AP-1 site, which is shown to be functional in IL-2 promoter activity, is next to the 3′ end of the CD28RE. The mutations in the CD28RE may somehow affect the cross talk between κB and AP-1 transcription factors as well as the regulation of the IL-2 gene. In regard to this question, we do not exclude the possibility that the κB element in the IL-2 promoter, under certain circumstances, is functional in vivo. Nevertheless, further studies are necessary to dissect the interrelationship between the κB and CD28RE motifs in activation of the IL-2 promoter.

Interestingly, we find no synergistic effect between RelA (p65) and Raf-1, or RelA (p65) and anti-CD28 MAb, stimulation in the wild-type IL-2 promoter (37a), which suggests that regulation of the IL-2 promoter is more tightly controlled than that of the minimal CD28RE site outside the context of the IL-2 promoter. These findings also provide a possible explanation for the discrepancy between results obtained with the minimal κB element and results obtained with the κB element in the context of the IL-2 promoter (18, 63). We suggest that the synergism demonstrated by using the CD28RE-CAT reporter may require additional signals when the full-length IL-2 promoter is studied. However, the reporter construct containing the minimal enhancer is still very useful for examining its regulation and for identifying the signaling events. We are currently looking for the additional signals required for these synergistic interactions in the context of the IL-2 promoter.

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J.-H. Lai and G. Horvath contributed equally to the work presented in this report.

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